Stable transformation of BY-2 cells

Introduction

- Stably transformed BY-2 cells are generated by co-cultivation with *Agrobacteria tumefaciens* (strains GV3101, LBA4404 or EHA105)
- Adapted from: Graumann K and Evans DE, (2014), "Dynamics of the Plant Nuclear Envelope during Cell Division", Methods in Molecular Biology

Materials

1. LB medium with and without appropriate filter-sterilized bacterial selection antibiotic
2. Acetosyringone (3',5'-Dimethoxy-4'-hydroxyacetophenone) as 100 mM stock in ethanol
3. Sterile antibiotic stock solutions
   Timentin (ticarcillin disodium/ potassium clavulanate) and carbenicillin should be used to kill agrobacteria after co-cultivation. The working concentration of these antibiotics is as follows: 20 mg/l timentin, 100 mg/l carbenicillin. Antibiotics are also required for selection of transformants. The choice of the antibiotics is dependent on the vector used but can include kanamycin and hygromycin B. Working concentrations for these two are as follows: 100 mg/l kanamycin, 40 mg/l hygromycin B.
4. *Agrobacterium tumefaciens* strains GV3101 or EHA105 or LBA4404 transformed with vector containing appropriate construct should be used.
5. BY-2 suspension culture (3-day-old; prepared as described above)
6. Sterile liquid BY-2 medium (approximately 70 ml per construct)
7. Sterile solid BY-2 medium plates without antibiotics (1 plate per construct) and plates with selection antibiotic, 100 mg/l carbenicillin and 20mg/l timentin (10 plates per construct)
8. Stationary incubator at 25^{o}C, no light
9. Shaking incubator, 25^{o}C set to 130 rpm, no light
10. Sterile pipette tips and pipettes
11. Sterile 1.5 ml microcentrifuge tubes (1 per construct) and 15ml sterile centrifuge tube (2 per construct)
12. Forceps, sterile
13. Laminar flow hood
14. Tin foil
15. Parafilm
16. Benchtop microcentrifuge

17. Coldroom or refrigerator

Method

Growing agrobacteria

Inoculate 5 ml LB medium (containing appropriate filter-sterilized bacterial selection antibiotic) with a single colony of *Agrobacterium tumefaciens* GV3101 or EHA105 or LBA4404 strain transformed with a binary vector containing the appropriate construct. Alternatively, use swab of glycerol stock for inoculation. Incubate 16 to 20 h at 180 rpm and 28°C.

Stable transformation of BY-2 cells

Use a 3 d old BY-2 suspension culture (either wild type for single transformation or transformed line for co-expression)

Prepare agrobacteria as follows:

1. Centrifuge 1 ml liquid overnight agrobacteria cultures at 5 000 *g* for 5 min at RT. In a laminar flow hood or by the flame, remove supernatant and resuspend cells in 1 ml LB medium containing acetosyringone (add 20 µl acetosyringone stock to 10 ml medium). Repeat centrifugation to finish first wash step. Repeat wash step two more times. After third wash, resuspend cells in 1 ml LB medium containing acetosyringone and incubate for 1 h at 4°C.
2. In a laminar flow cabinet, transfer 7 ml of the 3 d BY-2 culture to a 15 ml sterile tube, add 1.2 µl acetosyringone and 100 µl of prepared agrobacteria. Gently invert tube several times to mix cultures and then pour onto plate containing solid BY-2 medium with no antibiotics.
3. Seal plate with micropore tape (SLS) and either wrap in aluminium foil or place in a blacked out box, and incubate for 3 d at 25°C in the dark without shaking
4. After incubation, in laminar flow hood, transfer BY-2 cells from plate for washing by gently tapping the petri dish to loosen cells and then rinsing cells off the plate with 5-10 ml liquid BY-2 medium; transfer to sterile 15ml tube.
5. Wash the BY-2 cells three times with 15 ml liquid BY-2 medium containing 100 mg/L carbenicillin and 20 mg/L timentin; for each wash step, centrifuge the cells at 3000 rpm for 5 min (set breaks and acceleration to 0), remove supernatant and resuspend in

approximately 10 ml medium (final volume 15 ml). Alternatively to centrifugation, cells can be left for 10 min to settle naturally before removing supernatant. However, centrifugation causes fewer cells to be lost.

6. After the final wash step resuspend cells to a total of 10 ml. Transfer 1 ml of resuspended cells onto a plate containing solid BY-2 medium, timentin, carbenicillin and appropriate selection antibiotic.
7. Gently rotate plate to spread cells over the surface of the solid medium.
8. Seal plate with Parafilm and incubate in the dark at 25°C without shaking for 4-6 weeks until calli appear.
9. If constructs are fluorescent, use fluorescence stereomicroscope to screen the calli. Transfer calli to suspension as detailed in method for producing BY-2 suspension culture.
10. Subculture cells for at least 4 weeks before other downstream applications.
11. For double-transformation, BY-2 cells are first transformed with one construct and, once stably expressing suspension cells are established, these may be used for a second transformation with the other construct.